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Early Onset of CD8 Transgene Expression Inhibits the Transition from DN3 to DP Thymocytes

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In this paper we show that the effects of transgenic coreceptor expression on thymocyte development depend on the onset of transgene expression. Thus, a CD8 transgene expressed on CD44+CD25− (DN2) and CD44+CD25+ (DN3) cells causes a partial block at the stage when TCRβ selection takes place and diminishes expansion at the subsequent developmental stages, resulting in increased DN3 and markedly reduced double-positive (DP) thymocyte numbers. This effect is evident on a polyclonal TCR repertoire as well as in TCR-transgenic mice (F5). By contrast, a CD8 transgene that leads to the same degree of overexpression on DP thymocytes, but is not expressed on double-negative subsets, has no effect on thymus size or composition. Therefore, the reduction of DP thymocyte numbers in CD8 TCRtg mice can be attributed to interferences at early developmental stages rather than to increased negative selection of DP cells. *The Journal of Immunology*, 2000, 165: 1236–1242.

During their development, thymocytes pass through a well-defined sequence of phenotypic changes and undergo a number of selection processes. Precursors appear to enter the thymus as CD4−/low c-kit+ cells (1) and subsequently develop into CD4−CD8− double-negative (DN3) thymocytes. These cells are further subdivided by their surface expression of CD44 and CD25 (2–4). Thus, the most immature DN cells are CD44+CD25− (DN1) and develop via CD44+CD25+ (DN2) into CD44+CD25− (DN3) thymocytes. At this stage, the β locus of the TCR is rearranged and tested for functionality by pairing to the pre-Tα-chain (5, 6). In the case of productive β rearrangement and successful formation of the pre-TCR complex, thymocytes can pass the β selection step, enter the cell cycle to expand (7), down-regulate CD25, and develop into CD44−CD25− (DN4) cells and then into CD4−CD8+ double-positive (DP) thymocytes. Further positive and negative selection processes are determined by the specificity of the TCRαβ on DP thymocytes that develop into either CD4−CD8+ or CD4+CD8+ single-positive mature thymocytes.

The importance of the β selection step is exemplified by the profound block of thymocyte development at the DN3 stage in a range of mice deficient in components of the pre-TCR complex, such as TCRβ (8), pre-TCα (6), the CD3 chains (reviewed in Ref. 9) or in mice unable to rearrange the TCR α- and β-chain genes due to lack of Rag-1 or Rag-2 genes (10, 11). Mice deficient in signaling molecules such as Lck (12), SLP76 (13, 14), or LAT (15), mice overexpressing a kinase-inactive Lck (16) and the Zap-70−/Syk−/ (17) or Fyn−/Lck−/ (18, 19) double knockouts show a similar developmental block at the DN3 stage. This strongly suggests that these molecules are involved in downstream signaling through the pre-TCR complex necessary for efficient β selection, subsequent expansion, and transition to the next developmental stage.

Conversely, reintroduction of crucial components as transgenes into knockout mice, such as a rearranged TCR β-chain into Rag-2−/− mice (20) or constitutively active Lck into Rag-2−/− or pre-TCα-deficient mice (21, 22), or induction of a CD3 mediated signal by injection of antiCD3 Abs (23, 24) allows the release of the β selection block and at least partial reversal of the phenotype (for review, see Refs. 9 and 25–27).

One of the effects of pre-TCR signaling is the inactivation of p53, thus ensuring survival of β-selected cells, and the release of the cell cycle block, allowing for the proliferative burst observed between the DN3 and DP stages (28). A comparison between Lck−/− single-deficient and CD3−/−/Lck−/− double-deficient mice showed that the latter mice have a further reduction in DP cells compared with the lck−/− mice due to impaired proliferation in those DN3 cells that have already passed β selection (29). Thus, poor generation of DP thymocytes in the double-deficient mice appears to be a combined effect of inefficient β selection and reduced proliferative burst at the transition to DP cells, indicating that the signaling requirements of these two events are different (29).

In DP and mature single-positive T cells, it is thought that during the interaction of TCR with MHC/peptide complexes, the role of the coreceptors CD4 and CD8 is to recruit Lck into the TCR complex and thus allow phosphorylation by Lck of the ITAMs (immunoreceptor tyrosine-based activation motifs) contained in the CD3 chains. Because thymic expression of coreceptor transgenes is a common tool in studies of thymocyte development, this raises the question of how these transgenes may interfere with the Lck-mediated signal required for successful β selection and subsequent proliferation.

To study these effects we compared two different CD8 transgenic mouse lines: mice transgenic for a genomic fragment containing the CD8 β-chain gene coexpressed with the mouse CD8α/ Lyt2.1 chain gene expressed under the control of the human (h) CD2 promoter (CD8tg) (30) and mice expressing the genomic P1−5 fragment that encompasses both CD8α and CD8β genes together with 2 kb 5′ of CD8β and ~25 kb 3′ of CD8α (P1−5) (31).
CD8tg mice show constitutive expression of the CD8 transgene on all T cells, including CD4 and CD8 single-positive cells. The latter fact made CD8tg mice a useful tool in studies of lineage commitment where forced expression in the CD4 lineage was required (30, 32–34). In contrast, the expression of the P1–5 transgene is restricted to DP thymocytes and CD8 single-positive T cells, closely following the pattern of endogenous CD8 expression. The effects of the two CD8 transgenes were compared in the situation of a polyclonal TCR repertoire as well as in mice expressing a transgenic TCR (F5) that recognizes the influenza virus nucleoprotein-derived peptide NP68 in the context of H-2D^d (35).

In this paper we report that in CD8tg mice, precocious CD8 transgene expression in the thymus causes a partial block of β selection and a decrease in subsequent expansion, leading to reduced numbers of DP thymocytes. This effect is not seen in P1–5 mice, in which onset of transgene expression is later but total CD8 levels on DP thymocytes are similar to those in CD8tg mice. Previous publications have suggested that transgene-driven overexpression of CD8 in DP may increase overall avidity and therefore lead to increased elimination of DP thymocytes by negative selection (36, 37). Here we show that a critical factor determining CD8 transgene-associated reduction of DP thymocyte numbers is the onset of CD8 transgene expression.

Materials and Methods

Mice

Mice transgenic for the F5 TCR (35) or for the genomic CD8 fragment P1–5, encompassing the CD8αα/Lyt2.2 and CD8 β-chain (31), were generated in our laboratory. Mice transgenic for a genomic fragment containing the CD8 β-chain gene coinjected with the mouse CD8αα/Lyt2.1 chain gene expressed under the control of the IκB2 promoter (30) were provided by Dr. Ellen Robey. Mice deficient in the expression of Rag-1 (38) were a gift from Dr. Eugenia Spanopoulou. To distinguish between endogenous and transgenic CD8 expression, CD8tg mice are backcrossed to C57BL/10 mice and therefore express the transgenic CD8αα/Lyt2.1 allele and endogenously the CD8αα/Lyt2.2 allele. Conversely, P1–5 transgenic mice were made in the CBA/Ca strain and thus express the transgenic CD8αα/Lyt2.2 allele and endogenously the CD8αα/Lyt2.1 allele. All transgenic mice in this study are heterozygous for the respective transgene(s). The mice were kept in a conventional animal colony free of pathogens and were analyzed at 6–8 wk of age if not otherwise indicated.

Flow cytometry

For flow cytometric analysis, 10^6 cells were stained with the following mAbs and second layer reagents: APC-conjugated anti-CD4 (PharMingen, San Diego, CA), PE-conjugated anti-CD4 (Sigma, St. Louis, MO), Tricolor-conjugated anti-CD4 (Caltag, Burlingame, CA), FITC-conjugated anti-CD8αα/Lyt2.1 (clone 49-11.1, PharMingen), biotin-or FITC-conjugated anti-CD8αα/Lyt2.2 (clone 2.43) (39), Tricolor-conjugated anti-panCD8α (Caltag), biotin-conjugated anti-CD25 (clone 7D4, Pharmingen), PE-conjugated anti-CD4 and CD44 (clone IM7, PharMingen), streptavidin-conjugated RED 670 (Life Technologies, Paisley, U.K.), or APC (Molecular Probes, Eugene, OR). Samples were analyzed on a FACScan or FACS Vantage flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson).
normal mice. Thus, our results indicate that increased numbers of DN3 cells and reduced numbers of DP cells in CD8tg mice may be caused by a partial block at the stage of $\beta$ selection and a reduced subsequent proliferation.

In mice deficient for components of the pre-TCR, impaired $\beta$ selection and a reduction in subsequent expansion have been associated with increased expression levels of CD25 on DP thymocytes (6, 40). Since we observed a partial block in $\beta$ selection, we checked CD25 expression levels on DP thymocytes in the CD8 transgenic mice. Fig. 4 shows that DP thymocytes from CD8tg mice have increased levels of CD25 expression compared with those from B10 mice, further suggesting that reduced DP cell numbers in these mice are associated with impaired $\beta$ selection and expansion. In contrast, DP cells from P1–5 mice express the same levels of CD25 as those from normal CBA mice, indicating that progression from the DN through the DP stage of development is unaffected by CD8 overexpression in P1–5 mice.

The observed block at the $\beta$ selection stage in combination with reduced clonal expansion could be caused by precocious expression of the CD8 transgene. Therefore, we compared expression levels of the CD8tg and P1–5 transgenes on DN subsets. Fig. 5 shows that in CD8tg mice, CD8 expression is switched on most DN2 and DN3 thymocytes (Fig. 5A), whereas these subsets are largely negative for transgenic CD8 expression in P1–5 mice (Fig. 5B). Similarly, DN4 thymocytes in the CD8tg mice are positive for transgene expression, whereas the DN4 subset in P1–5 mice is negative (not shown). Thus, the effect of the CD8tg transgene on thymocyte development appears to correlate with early onset of expression in thymocyte subsets undergoing $\beta$ selection and subsequent expansion.

The partial block in $\beta$ selection described above could be caused by a block in endogenous TCR$\beta$ gene rearrangement. To determine whether the effect is still observed in the presence of rearranged TCR chains, we used mice transgenic for an MHC class I-restricted TCR (F5) (35) and bred F5/Rag-1$^{−/−}$ to CD8tg mice. Thymuses from F5/Rag-1$^{−/−}$ mice and F5/Rag-1$^{−/−}$/CD8tg mice were analyzed for cellularity and by FACS for DP and DN1-DN4
thymocyte subset distribution (Fig. 6A). Similar to the situation in polyclonal mice, CD8tg expression in F5/Rag-1<sup>−/−</sup> mice leads to a reduction in total and DP thymocyte numbers in combination with an increase in the percentage and absolute numbers of DN cells. In contrast to F5/Rag-1<sup>−/−</sup> mice, which do not generate CD4<sup>+</sup>CD8<sup>−</sup> cells, a large number of these cells are seen in the thymus of F5/Rag-1<sup>−/−</sup>/CD8tg mice. The development of CD8-expressing CD4<sup>+</sup>CD8<sup>−</sup> single-positive cells that are rescued by constitutive expression of the CD8 transgene in these mice has been described and discussed previously (33, 34). Further analysis of DN thymocytes reveals that the DN3 subset is over-represented in F5 mice expressing the CD8 transgene (Fig. 6A). As in the polyclonal situation, CD25 expression levels on the DN3 subset are higher in F5/CD8tg mice than in F5 mice. In absolute numbers, the thymuses of F5/CD8tg mice show a 3-fold increase in DN3 and a 6-fold decrease in DP cell numbers compared with F5 mice (Fig. 6B), suggesting that CD8tg expression leads to a partial block at the β selection step and decreased subsequent expansion, as in polyclonal B10/CD8tg mice. Similar to these mice, CD25 expression is increased on DP thymocytes from F5/CD8tg mice compared with that in F5 mice (Fig. 6A), further confirming the association between low DP numbers and incomplete DP development caused by CD8tg expression. As in B10/CD8tg mice, the majority of DN2, DN3, and DN4 cells express the CD8 transgene in F5/Rag-1<sup>−/−</sup>/CD8tg mice (not shown). In summary, it appears that the effect of CD8tg expression is not associated with a block in TCRβ gene rearrangement, as it is seen in both the presence and the absence of a rearranged TCR β-chain.

As mentioned above, P1–5 transgenic mice show no change in the distribution and cell numbers of thymocyte subsets. Thus, CD8 overexpression in DP thymocytes appears not to lead to increased deletion of DP cells. On a polyclonal background, however, increased negative selection of some TCRs by increased overall avidity could be compensated by positive selection of clones with lower affinity TCRs. Therefore, we decided to analyze the effect of P1–5 expression in the situation of a monoclonal population with fixed TCR, using F5/Rag-1<sup>−/−</sup> mice and F5/Rag-1<sup>−/−</sup>/P1–5 mice. A comparison of thymuses from age-matched 4-wk-old mice shows that absolute numbers of total and DP thymocytes are unaltered, while percentage and numbers of DN cells are reduced in F5/Rag-1<sup>−/−</sup>/P1–5 mice, suggesting that β selection and subsequent expansion proceed efficiently (Fig. 7). Similarly, analysis of thymuses from adult mice shows no difference in total or DP thymocyte numbers (not shown). Maturation of CD4<sup>+</sup> single-positive cells is not observed in F5/Rag-1<sup>−/−</sup>/P1–5 mice. This is due to the lineage-specific expression pattern of the P1–5 transgene, which is switched off in CD4 SP cells and therefore cannot rescue F5-expressing thymocytes developing into the CD4 lineage. These results indicate that CD8 overexpression in DP thymocytes from F5/Rag-1<sup>−/−</sup>/P1–5 mice does not lead to increased elimination of these cells.

In conclusion, our data show that early onset of CD8 transgene expression in DN2 cells causes a partial β selection block and a decrease in subsequent proliferative expansion, leading to strongly reduced DP thymocyte numbers. Because both CD8tg and P1–5 transgenes cause similar CD8 overexpression on DP cells, but P1–5 does not alter the percentage or number of DP thymocytes, overexpression of total CD8 on DP thymocytes does not lead to increased elimination of these cells.

**Discussion**

This study shows that CD8 expression on DN2 and DN3 thymocytes interferes with β selection and subsequent expansion of thymocytes. Thus, in CD8tg mice thymus cellularity and the percentage of DP cells are reduced, while the proportion and numbers of DN3 thymocytes are increased. These effects are clearly dependent on the early expression of the CD8 transgene, since the P1–5 transgene, which closely follows the endogenous CD8 expression pattern, has virtually no effect on thymocyte number or composition.

**Figure 4.** CD8 overexpression by the CD8tg, but not by the P1–5 transgene, leads to increased CD25 expression levels on DP thymocytes. Flow cytometric analysis of thymuses from mice with the indicated genotypes. Thymocytes were triple stained for CD4, endogenous CD8, and CD25, and histograms show CD25 expression of DP thymocytes electronically gated as indicated in Fig. 2A. The mean fluorescence intensity is annotated in the histogram. Given the strain background and CD8a allele encoded by the transgene, endogenous CD8 expression is detected by an anti-CD8a/Lyt2.2 mAb in B10 and B10/CD8tg mice and by an anti-CD8a/Lyt2.1 mAb in CBA and CBA/P1–5 mice.

**Figure 5.** Expression pattern of the CD8tg and P1–5 transgenes on DN thymocyte subsets. Flow cytometric analysis of transgenic CD8 expression on DN thymocyte subsets. A and B, Transgenic CD8 expression on CD44<sup>−</sup>CD25<sup>−</sup> (DN2) or CD44<sup>−</sup>CD25<sup>+</sup> (DN3) thymocytes from the following mice: A, (CBA × B10)F<sub>1</sub> (shaded plot) and B10/CD8tg (bold line); or B, (CBA × B10)F<sub>1</sub> (shaded plot) and CBA/P1–5 (bold line). Total thymocytes were stained for the expression of CD4 and CD8end with mAbs bound to the same fluorochrome, for CD25, CD44, and transgenic CD8. Histograms show cells gated first on the CD4<sup>+</sup>CD8<sup>−</sup>− DN population and subsequently on the CD44<sup>−</sup>CD25<sup>−</sup> (DN2) or the CD44<sup>−</sup>CD25<sup>+</sup> (DN3) subset as shown in Fig. 3. The percentages of CD8<sup>−</sup>− cells are indicated for the transgenic mice. Given the strain background and CD8a allele encoded by the transgenes, transgenic CD8 expression is detected by an anti-CD8a/Lyt2.1 mAb in A and by an anti-CD8a/Lyt2.2 mAb in B. (CBA × B10)F<sub>1</sub> were chosen as controls because they express both CD8a/Lyt2.1 and CD8a/Lyt2.2 endogenously.
despite a comparable 2-fold higher level of total CD8 expression on DP thymocytes.

Würch et al. have shown that β selection and subsequent expansion are discernible events that may have different signaling requirements (29). The effect we observe appears to act on both events, since DN3 numbers are increased, on the one hand, indicating that cells accumulate at the pre-β selection stage. On the other hand, whereas the absolute number of the DN4 subset is unaltered, there is a marked reduction in the number of DP thymocytes that derive from them. This suggests an additional effect of the expression of the CD8tg on the expansion during the DN4-DP transition observed in normal animals. Thus, as described in other systems, poor generation of DP thymocytes in CD8tg mice seems to be a combined effect of inefficient β selection and reduced expansion in the following phases.

The transgenic expression of the CD4 or CD8 coreceptor molecules in the thymus has been widely used as a tool for the analysis of lineage commitment and to test models of positive and negative selection. In particular, the decrease in the number and proportion of DP thymocytes caused by CD8 overexpression was taken as evidence for increased negative selection (30, 36). Here we show that precocious expression of coreceptor transgenes can interfere with thymocyte development at a very early stage, causing a reduction in the number of DP thymocytes that derive from them. This suggests an additional effect of the expression of the CD8tg on the expansion during the DN4-DP transition observed in normal animals. Thus, as described in other systems, poor generation of DP thymocytes in CD8tg mice seems to be a combined effect of inefficient β selection and reduced expansion in the following phases.

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It appears that if onset of CD8 transgene expression mimics as closely as possible the endogenous expression pattern as in P1–5 transgenic mice, then no effects on early developmental stages are observed. This transgenic expression pattern allowed us to study the effects of coreceptor overexpression on the selection of DP thymocytes. In the F5/P1–5 mice the affinity of the F5 TCR for natural ligands combined with 2-fold CD8 levels appear to lead to an overall avidity that remains below the threshold for negative selection. However, results from other groups showing that CD8 overexpression may increase negative selection could depend on differences in TCR affinity and the degree of CD8 overexpression in the particular CD8 transgenic line used (36) as well as on the different onsets of transgene expression.

It was possible that early CD8 expression would interfere with TCRβ gene rearrangement, which, in turn, would prevent formation of a pre-TCR complex and, therefore, β selection. To exclude this possibility we used F5 TCR transgenic mice, thus providing a rearranged TCR β-chain. Since we saw the same effect of CD8tg expression in F5 mice as in B10 mice, it appears that the β selection block is not caused by a block in TCRβ rearrangement. We
can also exclude the possibility that transcription of essential endogenous genes or the F5 transgene is suppressed by competition of the hCD2 expression cassette for transcription factors, since F5 mice containing 50 additional copies of the hCD2 cassette or other transgenic mice expressing irrelevant genes under the control of the same cassette show no alteration in thymocyte size or composition (data not shown).

One of the central functions of coreceptor molecules in T cells is the recruitment of the Src family kinase Lck into the TCR signaling complex. As Lck-deficient mice show a partial block in β-selection, Lck-mediated signals appear to be important in this process. In fact, the similarity between coreceptor-mediated signals and those required for β selection was demonstrated by Norment et al. (41), who showed that expression of a CD4 transgene is in DN3 cells (42), the presence of another protein expression is in DN3 cells (42), the presence of another.

**References**


